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TITLE: Prostasin Serine Protease as a Breast Cancer Invasion Marker and a Metastasis Suppressor

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
We have previously shown that membrane-anchored prostasin serine protease was an *in vitro* suppressor of tumor cell invasion (1-3). The expression of prostasin is down-regulated in prostate cancers (2), and invasive cancer cell lines of the human prostate and breast (1, 3). We hypothesized that the down-regulation of prostasin is causal to breast cancer invasion and metastasis *in vivo*. Two highly invasive human breast cancer cell lines, the MDA-MB-231 and MDA-MB-435, were transfected with a prostasin cDNA plasmid to restore prostasin expression. The transfectants of MDA-MB-231 were used in the nude mice model to assess the metastatic potential. The results demonstrated a statistically significant difference of metastasis between cells that express prostasin and the control cells. Prostasin expression in the transfected cells was analyzed by immunocytochemistry and clones that express prostasin more uniformly were obtained for repeat experiments in nude mice for the purpose of maximizing the effect of prostasin.

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INTRODUCTION

Prostasin Serine Protease as A Breast Cancer Invasion Marker and A Metastasis Suppressor.
Karl X. Chai, Ph.D., Idea Award

We have previously shown that membrane-anchored prostasin serine protease was an *in vitro* suppressor of tumor cell invasion (1-3). The expression of prostasin is down-regulated in prostate cancers (2), and invasive cancer cell lines of the human prostate and breast (1, 3). For this project, we hypothesized that the down-regulation of prostasin is causal to breast cancer invasion and metastasis *in vivo*. Two highly invasive human breast cancer cell lines that have been used previously by others to evaluate breast cancer metastasis *in vivo* (4), the MDA-MB-231 and MDA-MB-435, were shown not to express prostasin due to promoter DNA methylation (3). The experimental design for testing our hypothesis was to restore prostasin expression *via* plasmid DNA transfection into these two cell lines and to use the nude mice model to assess the metastatic potential of transfected cells that now express prostasin.

BODY

Task 1. Evaluation of prostasin as a breast cancer invasion/metastasis marker (Months 1-36).
Task deleted per recommendation by the Programmatic Review.

Task 2. Evaluation of prostasin as a therapeutic agent for breast cancer metastasis (Months 1-36).

1). Investigation of prostasin's role in controlling spontaneous metastasis of human breast cancer in animal model: Injection of human breast cancer cells MDA-MB-435/Pro (expressing recombinant human prostasin) or MDA-MB-435/Vec (vector-transfected control) into the mammary fat pad of nude mice
Months1-6: Establishment of sublines MDA-MB-435/Pro and MDA-MB-435/Vec, and *in vitro* testing of invasiveness, using cell lines provided by Dr. J. E. Price

Months 7-36: Three repeat experiments are planned for the 12-week with-tumor study of prostasin's effect on spontaneous metastasis

Progress: The MDA-MB-435 cell line was received from Dr. Price, cultured and tested in the laboratory for prostasin expression. We have confirmed a lack of prostasin protein or mRNA expression in this cell line by methods previously described (3) (data not shown), similar to what we had observed for the sub-line, the MDA-MB-435s (3). The MDA-MB-435 cell line was then transfected with the prostasin cDNA plasmid as described to obtain a mixture of ~100 colonies (3). Expression of prostasin protein in the mixed colony was confirmed by western blot analysis (data not shown). We then further evaluated the expression of prostasin protein in the mixed transfectants by immunocytochemistry using a previously described method (1). The result showed a varying level of prostasin protein expression in the transfected cells, approximately 50% of the cell population produced an intense immunostaining for prostasin (data not shown). This observation was quite different from that with the 293/Pro cells (1), in which ~100% of the mixed population of cells express the prostasin protein in a uniformly strong pattern. As our recombinant protein expression in the cancer cell lines was established with an episomal mechanism (2,3) and the cells are intended for injection into animals for an observation period

of ≥12 weeks, we decided to screen for clones with the most intense prostasin staining before commencing the animal experiments.

We then set up a new round of transfection with the control plasmid vector, and the prostasin cDNA plasmid. For each transfection, the previously described procedures were followed but the transfectants were cultured in 100-mm dishes with 800 μg/ml G418 for three weeks, or until colonies sized between 2-8 mm in diameter appeared. To pick the colonies, the culture media was removed and the cells were washed once with 1x PBS (pH 7.4). Single colonies were dislodged with the end of 200-μl pipette tips filled with 50 μl of warm trypsin (0.25% with 1 mM EDTA). The colonies were then withdrawn into the pipette tip and mixed on a sterile surface with pipeting motion, before they were cultured in 24-well plates with 1 ml of media containing G418 (800 μg/ml). Once the cells grew to confluence in the wells, they were trypsinized and placed onto glass coverslips for immunocytochemistry (using 10% of the cells). The remaining cells are expanded to larger culture areas. In Figure 1, we show the results of the immunocytochemical staining for prostasin in two independently selected colonies/clones, which are representative of the types of clones we have screened. Type-1 clones (upper panels) display an intense membrane signal of prostasin protein expression while ~100% cells are uniformly stained. For Type-2 clones (lower panels), only a very small percentage of cells display an intense membrane signal of prostasin protein expression while a great majority of cells are only stained weakly for prostasin expression.

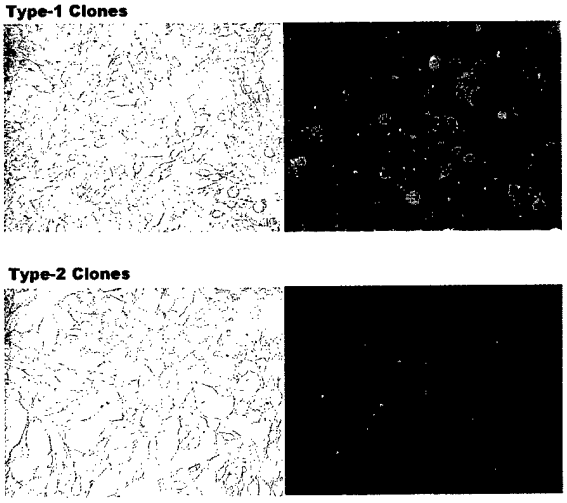

Figure 1. Immunocytochemical staining of membrane prostasin expression in transfected MDA-MB-435 cells. For each individual G418-resistant clone, 20,000 cells were cultured on a glass coverslip over night at 37°C and 5% CO_2 in a moist chamber. The cells were then washed in 1x PBS (pH 7.4), and fixed with 4% paraformaldehyde. No detergent was added during fixation. The cells were then subjected to prostasin immunostaining as described previously (1). The stained cells were viewed under a Zeiss Axioskop 2 equipped with a Sony DXC-950 camera and Zeiss Axiovision 3.1 software. The left panels show a bright-field image of the cells, while the right panels show the corresponding prostasin immunostaining where present (magnification: 400 x).

For mammary fat pad injection, we have selected six Type-1 clones and mixed at equal cell numbers. This mixture of Type-1 clones will be designated MDA-MB-435/Pro. From a separate transfection with the control plasmid vector, six independent clones were selected and mixed at equal cell numbers, and designated MDA-MB-435/Vec. The injection will be carried out in the immediate phase following this report.

2). Investigation of prostasin's role in controlling experimental metastasis of human breast cancer in animal model: Injection of human breast cancer cells MDA-MB-231/Pro (expressing recombinant human prostasin) or MDA-MB-231/Vec (vector-transfected control) into the tail vein of nude mice

Months 1-6: Establishment of sublines MDA-MB-231/Pro and MDA-MB-231/Vec, and *in vitro* testing of invasiveness, using cell lines provided by Dr. J. E. Price

Months 7-36: Three repeat experiments are planned for the study of prostasin's effect on experimental metastasis

Progress: The MDA-MB-231 cells received from Dr. Price were also confirmed for absence of prostasin protein and mRNA expression by previously described methods (3). Transfection was carried out to establish mixed colonies (~100) MDA-MB-231/Pro (expressing recombinant human prostasin) and MDA-MB-231/Vec (vector-transfected control). As these cells were assessed to be the same as those that were previously described, we by-passed the *in vitro* assays and directly proceeded to the animal experiments.

The animal experiments were carried out as proposed. Thirteen (13) female nude mice (5 and 1/2 weeks old) were used in the control group (receiving MDA-MB-231/Vec) as well as the prostasin group (receiving MDA-MB-231/Pro). Cells were cultured to near confluence, trypsinized, and washed with 1x HBSS. For each mouse, injection was carried out with a 27-gauge needle through the tail vein, using 3×10^6 cells, as described previously by Dr. Price (4). At four weeks following the injection, several animals appeared moribund and all animals were sacrificed. Lungs were removed from the animals for fixation and histological analysis. Both lungs from each animal were sectioned and stained with H/E for inspection of metastasized tumor. Representative sections of the two groups are shown in Figure 2, and Table 1 shows the average of metastatic loci in the two groups. The data of the two groups were analyzed by ANOVA (using Microsoft Excel), and the difference of metastatic loci between the two groups was statistically significant ($p < 0.05$).

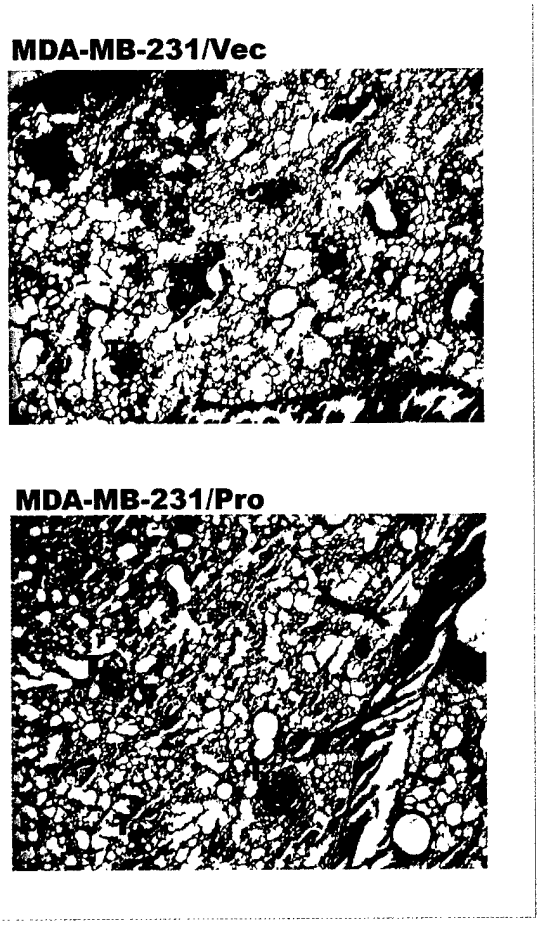

Figure 2. Histological staining of lung sections from nude mice injected with MDA-MB-231/Vec or MDA-MB-231/Pro. The tissues were removed, processed, and stained with H/E as described previously (2). For each animal consecutive sections were examined and those with the most metastatic loci were analyzed. The sections were viewed under a Zeiss Axioskop 2 equipped with a Sony DXC-950 camera and Zeiss Axiovision 3.1 software. The upper panel shows a representative section of the tissue from a mouse receiving the control cells (MDA-MB-231/Vec), and the lower panel shows that of an animal from the prostasin-treated group (MDA-MB-231/Pro) (magnification: 50 x). Metastatic tumor loci are marked with a boldface capital letter "T".

(Next page) Table 1. Metastatic loci (average) in mice receiving MDA-MB-231/Vec or MDA-MB-231/Pro. The data in each row represent the average metastatic loci in two separate areas of the tissues examined from each animal. For each group, 13 animals were used.

Table 1. Metastatic loci (average) in mice receiving:

Animals	MDA-MB-231/Vec	MDA-MB-231/Pro
	130	67
	75	42
	26	11
	3	3
	82	54
	96	72
	100	15
	74	63
	93	57
	122	56
	26	116
	100	30
	126	20

During the interim of the animal experiments using the MDA-MB-231 cells, the immunocytochemistry results on the MDA-MB-435 transfectants were obtained (see above). We then performed a similar experiment on the MDA-MB-231 transfectants (the MDA-MB-231/Pro), and found out that the mixed colonies show approximately 50% intense staining (similar to the Type-1 clones shown in Figure 1, data not shown). We have since performed transfection of MDA-MB-231 again, and identified clones that show uniform and intense prostasin staining (Type-1). In the repeat experiments we plan to use mixtures of six (6) independent clones for both the MDA-MB-231/Pro (Type-1) and the MDA-MB-231/Vec.

KEY RESEARCH ACCOMPLISHMENTS

- We have shown that prostasin re-expression in the MDA-MB-231 metastatic human breast cancer cell line can reduce the experimental metastasis to the lungs in nude mice.
- We have observed that the prostasin transfectants of human breast cancer cells, unlike the 293/Pro cells previously reported (1), do not express prostasin uniformly. Steps were taken to obtain mixed clones that express prostasin uniformly for use in animal experiments with long periods of observation.

REPORTABLE OUTCOMES

At this time, we have accumulated parts of the data that were anticipated, but no formal reports such as manuscripts have been prepared.

CONCLUSIONS

The progress in the first year of the planned research has confirmed our working hypothesis that prostasin is a potential metastasis suppressor of breast cancer *in vivo*.

"SO WHAT": Confirmation of an *in vivo* anti-metastasis role for prostasin serine protease will justify testing the potential of using prostasin or its interactive proteins as a therapeutic lead for patients who are already diagnosed with invasive breast cancer.

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APPENDICES

N/A